Lack of negative influence on the cellular transcription factors NF-κB and AP-1 by the Nef protein of human immunodeficiency virus type 1

Keejung Yoon and Sunyoung Kim

Institute of Molecular Biology and Genetics, Building 105, Seoul National University, Kwan-Ak-Gu, Seoul 151-742, Korea

In order to investigate the molecular mechanism of the reported negative effect of the Nef protein of human immunodeficiency virus type 1 (HIV-1) on the cellular transcription factors NF-κB and AP-1, human T cell lines (both populations and subclones) expressing the nef gene from HIV-1 clone pNL432 were constructed. Functional expression of the nef gene was confirmed by downregulation of CD4 and MHC class I proteins on the cell surface as measured by fluorescence-activated cell sorter analysis. However, contrary to previous reports, no significant difference was found in the induced level of NF-κB and AP-1 activity between nef+ and nef− cell lines upon stimulation by phorbol 12-myristate 13-acetate and phytohaemagglutinin, as measured by transient transfection and electromobility shift assays. These data indicate that the Nef protein does not have a negative effect on the induction of NF-κB and AP-1.

The Nef protein is encoded by an ORF that overlaps the 3′ LTR in human immunodeficiency virus type 1 (HIV-1), HIV-2 and simian immunodeficiency virus (SIV) (Allan et al., 1985; Beaver & Wong-Staal, 1989; Shibata et al., 1990). It is a myristoylated, membrane-associated protein of 27–34 kDa (Franchini & Robert-Guroff, 1986; Kaminchik et al., 1991). nef was originally so-named because it was an acronym for ‘negative factor’, but the bulk of data now support a positive role for nef during virus replication in vivo and in vitro (Chowers et al., 1994; de Ronde et al., 1992; Deacon et al., 1995; Kessler et al., 1991; Kim et al., 1989; Kirchhoff et al., 1995; Miller et al., 1994; Nabel & Baltimore, 1987).

The NF-κB family normally regulates the expression of genes involved in T cell activation and proliferation, such as interleukin 2 (IL-2) and the alpha subunit of the IL-2 receptor (IL-2R) (Greene, 1991). NF-κB is thought to play an important role in HIV-1 replication because the HIV-1 promoter contains two NF-κB binding sites (Nabel & Baltimore, 1987). It has been suggested that the Nef protein influences the activity of NF-κB. However, the nature of the interaction between these proteins appears to be controversial. It has been reported that the Nef protein may inhibit the induction of HIV-1 and IL-2-directed gene expression through interference with the NF-κB activation that occurs upon T cell stimulation (Bandres & Ratner, 1994; Niederman et al., 1992) and that the addition of recombinant Nef protein to peripheral blood mononuclear cells decreases T cell surface IL-2R alpha-chain expression (Greenway et al., 1995). Furthermore, it has been suggested that induction of AP-1 is inhibited in the presence of the Nef protein (Bandres & Ratner, 1994).

However, a positive role(s) for the Nef protein in T cell activation and HIV gene expression has also been proposed. The secretion of IL-2 and surface expression of IL-2R in response to phorbol 12-myristate 13-acetate (PMA) in Jurkat cells (Schwartz et al., 1992) and antigen-dependent T cells (Brigino et al., 1997) are unaffected by the presence of the Nef protein. Moreover, nef-containing SIV preferentially stimulates the production of IL-2 from 221 cells (Alexander et al., 1997). These cells are a T lymphoid cell line derived from a rhesus monkey infected with herpesvirus saimiri and are known to closely resemble normal lymphoid cells in that they produce IL-2 only upon stimulation. The positive effect of the Nef protein on HIV-1 replication is most evident when resting CD4+ T lymphocytes are infected by HIV-1 followed by stimulation with T cell mitogens such as phytohaemagglutinin (PHA) and PMA (Miller et al., 1994; Spina et al., 1994). Because of these conflicting reports, we initiated a series of experiments designed to help understand the role(s) of nef in viral gene expression. We found, reproducibly, that the Nef protein had little effect on the activity of the cellular transcription factors NF-κB and AP-1.

To study the effects of the Nef protein on the cellular transcription factors NF-κB and AP-1, we constructed cell lines that constitutively expressed the Nef protein using a retroviral vector. The nef coding sequence was isolated from the HIV-1 clone pNL432 by PCR, confirmed by nucleotide sequencing after cloning into the retroviral vector MOIN (Kim et al.,...
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Fig. 1. Detection of Nef protein and effects of Nef on induction of NF-κB and AP-1 by PHA and PMA. (a) Cell extracts were prepared from various lines and analysed by Western blotting using antibody specific to Nef (g2121; NIH AIDS Research and Reference Reagent Programme). The filled-in and open arrows indicate the 27 and 25 kDa forms of the Nef protein, respectively. Cells were transiently transfected with NF-κB test plasmid, J16, carrying a truncated promoter of c-fos containing κB binding sites driving CAT activity (b), or with an AP-1 CAT plasmid containing a CAT gene under the control of a truncated c-fos promoter with five copies of a synthetic AP-1 binding site (GGTACACTGGCCG) (c). Half of each cell culture was induced with PMA and PHA (filled-in bars), while the other half remained untreated (open bars). CAT activity was determined by conversion of [14C]chloramphenicol to its acetylated forms. Error bars show the standard deviations from a minimum of three independent experiments.

We first tested whether the Nef protein was expressed in these drug-resistant cells. The nef+ cell population (J-432 nef) and all subclones (J2-1 to J2-8) derived from this population expressed readily detectable amounts of the 27 kDa Nef protein (Fig. 1 a; filled-in arrow). The 25 kDa Nef protein (open arrow) was also detected in some lines. It has been suggested that the 25 kDa Nef protein is the translation product of a reading frame initiating at an internal ATG codon in the nef ORF, 57 bases downstream from the first ATG codon (Kaminchik et al., 1991). To be certain that nef sequences in these G418-resistant cells were still identical to that of NL432, nef sequences were cloned by genomic PCR from J2-1, J2-5 and J2-8, followed by nucleotide sequencing. Two sequences were cloned from each line. The nef sequences in these cell lines were all identical to that of the NL432 nef.

To our knowledge, the only properties of the Nef protein that are not in dispute are its inhibitory effects on cell surface CD4 and MHC class I molecules. To be certain that the Nef protein expressed in these cell lines was functional, we therefore measured levels of these markers on the cell surface by fluorescence-activated cell sorter (FACS) analysis. Relative to the parental Jurkat line, the level of CD4 was decreased by almost 40% in the population containing the nef coding sequence, while there was no change in the nef− control population (Table 1). The inhibitory effect of the Nef protein on CD4 was more pronounced in individual subclones. In these lines, the levels of CD4 were decreased by 30–97%. Consistent with previous reports, the downregulation of MHC class I was

protein, respectively. Cells were transiently transfected with NF-κB test plasmid, J16, carrying a truncated promoter of c-fos containing κB binding sites driving CAT activity (b), or with an AP-1 CAT plasmid containing a CAT gene under the control of a truncated c-fos promoter with five copies of a synthetic AP-1 binding site (GGTACACTGGCCG) (c). Half of each cell culture was induced with PMA and PHA (filled-in bars), while the other half remained untreated (open bars). CAT activity was determined by conversion of [14C]chloramphenicol to its acetylated forms. Error bars show the standard deviations from a minimum of three independent experiments.
less prominent than that of CD4. The nef<sup>+</sup> population showed a 10% reduction in MHC class I, while the magnitude of downregulation in the subclones varied from 0–60%. A reasonable correlation was found between the level of the magnitude of downregulation of these two surface markers. For example, clone J2-2 showed the lowest level of MHC class I as well as CD4 molecules.

To determine whether the Nef protein could affect the induction of NF-κB following external stimulation, nef-expressing cell lines were transfected with 10 μg reporter plasmid J16 carrying a truncated c-fos promoter with κB binding sites, using DEAE-dextran (Grosschedl & Baltimore, 1985; Kim <em>et al</em>, 1996). Following transfection, the cells were maintained in the medium for 24 h. Half of the transfected cells were treated with 13 μg/ml PHA-P and 75 ng/ml PMA (both from Sigma) and incubated for an additional 18 h (Niederman <em>et al</em>, 1992). Untreated cells were used to measure the basal activity of NF-κB. Cell extracts were prepared and CAT assays were performed by a standard procedure as previously described (Kim <em>et al</em>, 1998). Extracts were normalized for protein concentration as determined by Bradford reagent analysis (Bio-Rad). The percent conversion of [14C]chloramphenicol to its acetylated forms was determined by quantifying spots for acetylated or non-acetylated chloramphenicol with a phosphoimager (FUJIX BAS1000). In five independent experiments, we found that there was no significant difference in the level of NF-κB activity induced in the nef<sup>+</sup> and nef<sup>−</sup> populations (Fig. 1b). In the subclones, the level of NF-κB induction appeared to vary. Levels of CAT activity in five of the eight subclones expressing the Nef protein were comparable to or slightly higher than the nef<sup>−</sup> subclones. Surprisingly, three nef<sup>+</sup> subclones, J2-1, J2-5 and J2-8, produced 5- to 10-fold higher levels of CAT activity following PMA/PHA induction than the subclones derived from the nef<sup>−</sup> population. Similar results were obtained using 5- to 20-fold lower amounts of the reporter plasmid (data not shown). This high increase in CAT activity in the nef<sup>+</sup> lines was not due to differences in transfection efficiency, as measured by a lacZ reporter plasmid (data not shown). Overall, these results indicated that the Nef protein did not inhibit NF-κB induction by PMA/PHA, which is an experimental system commonly employed by other investigators to study the effect of the Nef protein.

Because the Nef protein was reported to have a negative effect on AP-1 induction as well as NF-κB (Bandres & Ratner, 1994), we also tested the effect of the Nef protein on induction of AP-1 by the same stimulators using the reporter plasmid AP-1 CAT. This contains a CAT gene under the control of a truncated c-fos promoter with five copies of a synthetic AP-1 binding site (Robbins <em>et al</em>, 1990). As in the case of NF-κB, there was no significant difference in the level of induced AP-1 activity between the nef<sup>+</sup> and nef<sup>−</sup> populations and subclones (Fig. 1c). However, the J2-1, J2-5 and J2-8 subclones which showed the highest induced levels of NF-κB also produced the highest levels of CAT activity following stimulation from the AP-1 reporter plasmid.

To test whether the transcriptional activity measured by the CAT assay correlated with DNA binding activity by these

### Table 1. FACS analysis of CD4 and MHC class I

Cells were stained with anti-CD4 or MHC class I antibody conjugated to FITC and their fluorescence was measured by flow cytometry. As a control, anti-mouse IgG antibody conjugated to FITC was used. The relative level was calculated by dividing the amount of CD4 or MHC class I protein in each cell line by that in the nef<sup>−</sup> cell population J-435 nef.

<table>
<thead>
<tr>
<th>nef status</th>
<th>Cell line</th>
<th>Relative amount of CD4</th>
<th>Relative amount of MHC class I</th>
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<tr>
<td>nef&lt;sup&gt;+&lt;/sup&gt;</td>
<td>J-432 population</td>
<td>62.9</td>
<td>89.4</td>
</tr>
<tr>
<td>nef&lt;sup&gt;−&lt;/sup&gt;</td>
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<td>90.2</td>
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<td>J2-2</td>
<td>3.0</td>
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<td>nef&lt;sup&gt;−&lt;/sup&gt;</td>
<td>J2-3</td>
<td>17.7</td>
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<td>J2-4</td>
<td>55.8</td>
<td>97.2</td>
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<td>22.0</td>
<td>98.4</td>
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transcription factors, nuclear extracts, either prior to or following stimulation with PHA and PMA for 4 h (Niederman et al., 1992), were prepared as described previously (Dignam et al., 1983). They were then incubated with a double-stranded $^{32}$P-labelled NF-κB oligonucleotide probe, the sequence of which was taken from the κB sites of the HIV-1 LTR (−99 to −76) (Kim et al., 1996), or an AP-1 oligonucleotide probe (Kekule et al., 1993). The non-inducible transcription factor Oct-1 was used as a control for the integrity of extracts. After incubation for 20 min at room temperature, the reactions were analysed on a 4% polyacrylamide gel. The specificity of the retarded complexes was confirmed by competition with a 50-fold excess of cold wild-type (Fig. 2a, b, c, lane 10) or mutant oligonucleotide (lane 11). Consistent with the CAT assay data, NF-κB induction by PHA and PMA was higher in the J2-1 subclone (Fig. 2a, lane 3) than in the J2-3 (lane 5), J5-3 (lane 7) and J5-4 (lane 9) subclones. The results using the AP-1 probe paralleled those obtained with the NF-κB oligonucleotide (Fig. 2b), though induction of AP-1 was less prominent than that of NF-κB in J2-1 cells. These data indicate that the induction of DNA binding activity of NF-κB and AP-1 correlates well with the increased CAT activity from the reporter plasmids.

Our data strongly indicate that the Nef protein does not have any negative effect on the ability of NF-κB to be induced in Jurkat cells following stimulation with PMA/PHA. There was no significant difference in the level of CAT activity obtained from reporter plasmids between nef$^+$ and nef$^-$ populations or in their subclones. The level of induced NF-κB varied widely in the different subclones; some clones expressing the Nef protein showed an induced level of NF-κB activity comparable to that observed in the nef$^-$ controls, while others showed dramatically increased activity. Differences in levels of NF-κB and AP-1 activity in various cloned lines might be the result of clonal variations during the subcloning procedure, which is commonly found when transformed lines are used in these types of experiments. Whatever the reason for clonal variation, if the Nef protein indeed suppressed the induction of these transcription factors, all nef$^+$ cells should have contained lower levels of activity of these transcription factors than nef$^-$ cells. There appeared to be no correlation between the activity of the κB reporter plasmid and the extent of CD4 downregulation by the Nef protein. For example, the J2-3 and J2-5 clones showed similar levels of CD4 downregulation but the levels of CAT activity from the reporter plasmid were significantly different. The same is also true for subclones J2-1 and J2-6. If the Nef protein affected the

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**Fig. 2.** Electromobility shift assay with extracts prepared from subclones. Nuclear extracts from J2-1, J2-3, J5-3 and J5-4 clones, either prior to (−) or following stimulation with PHA and PMA (+), were prepared and incubated with labelled oligonucleotide probe for (a) NF-κB, (b) AP-1 or (c) Oct-1. Specific DNA–protein complexes were verified by competition assay which involved incubating the protein sample from lane 3 with unlabelled wild-type (W) or mutated (M) oligonucleotide. S, Specific DNA–protein complex; FP, free probe.
induction of NF-κB, we might expect to see comparable levels of induced activity of these transcription factors.

The results presented here are in agreement with observations that nef expression in antigen-dependent T cell clones does not affect their proliferative responses to antigen, and other parameters of T cell activation, such as the induction of IL-2R alpha-chain expression and cytokine production (Page et al., 1997). Because we used the same nef sequence (NL432) and the human T cell line (Jurkat) that had been previously used by other investigators, we do not believe that the use of different experimental tools can account for these observed discrepancies. This is especially so because in all of our nef+ cell lines, we always observed a significant reduction in the level of surface CD4. Because the interaction between the Nef protein and NF-κB has been proposed to play such a key role in virus replication and pathogenesis, it is vital to clarify the relationship between these two proteins.

We thank Paula Cannon for critically reading the manuscript. This work was supported in part by the research grants from the Korea Research Foundation (S.K.), the Ministry of Science and Technology (08-02-02: S.K.) and the Korea Science and Engineering Foundation (96-0403-03-01-3: S.K.).

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Received 12 May 1999; Accepted 11 August 1999